Model for the Aerobic Growth of *Aeromonas hydrophila* K144

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ABSTRACT

The combined effects of temperature (5 to 42°C), NaCl (0.5 to 4.5%), pH (5.3 to 7.3), and $NaNO_2$ (0 to 200 μg/ml) on the aerobic growth of *Aeromonas hydrophila* K144 were studied in brain heart infusion (BHI) broth using a modified central composite design. Variable combinations were tested in triplicate aerobic flasks; viable cell counts were made at intervals during incubation by surface plating on tryptic soy agar. Growth curves were generated using the Gompertz equation in conjunction with a nonlinear iterative regression analysis. Values for the four Gompertz parameters (A, C, B, and M) were obtained for the variable combinations tested. Using response surface techniques, quadratic and cubic equations containing the four variables of temperature, pH, NaCl, and $NaNO_2$ were developed to yield predictive values for the B and M Gompertz values. Goodness of fit evaluation of the models was by R^2 values. Comparison of predicted and observed values of B and M and evaluation of predicted lag times and generation times indicated that the quadratic model gave a better fit. Overall, the variable combinations interacted to decrease the generation time and increase the lag time. The results indicate that pH, salt, and nitrite can decrease the growth of *A. hydrophila* when combined with low temperature incubation.

Organisms of the *Aeromonas* group (motile Aeromonads, *Aeromonas hydrophila* group, mesophilic Aeromonas) have received recent recognition as a foodborne pathogen of concern to the food industry, public health officials, and consumers. The organism occurs widely in the environment, particularly in various water supplies. *A. hydrophila* also has been isolated from different foods, including vegetables (*3*), water (*6*), and foods of animal origin (*8*). In a food survey by Palumbo et al. (*8*), A. *hydrophila* was detected in virtually every sample of fish and seafood, red meat, and poultry examined. In addition to its detection in every sample, the organism increased in number during one week's storage at 5°C. This last observation supports the literature (*7*) that indicates *A. hydrophila* is one of a group of foodborne pathogens capable of growth at 5°C, a temperature formerly thought adequate to keep food safe from foodborne pathogen hazards.

Consumers are currently demanding foods that are given less processing and contain fewer additives. This places increased emphasis on refrigeration as a means of restricting the growth of foodborne pathogens as well as spoilage microorganisms. However, as indicated above, there are foodborne pathogens which can grow at 5°C. Thus, inhibition of various foodborne pathogens depends on the interaction of factors such as NaCl, pH, and $NaNO_2$ along with low temperatures. This multifactorial approach to the study of growth kinetics and inhibition has proven useful with bacteria such as *Shigella flexneri* (Zaika et al., in preparation), *Listeria monocytogenes* (*1,2*), *Clostridium botulinum* (*4*), and *Salmonella* (*5*).

The purpose of this study was to investigate the combined effects and interactions of temperature, pH, NaCl, and $NaNO_2$ on the kinetics of aerobic growth of *A. hydrophila* in brain heart infusion (BHI Difco, Detroit, MI) broth with the goal of developing a model that could be used to predict the growth of the organism in any combination of the variables. This work extends a limited, earlier study of the influence of various factors on the aerobic growth of the organism (*9*).

MATERIALS AND METHODS

Organism

Aeromonas hydrophila K144 was used throughout these studies. All experiments were inoculated from a starter flask; the starter flask was prepared by inoculating 50 ml of BHI in a 250-ml flask and incubating overnight (18 to 20 h) at 28°C. Dilutions of the starter flask (made in 0.1% peptone water) were used to inoculate the experimental flasks. The count at zero time of incubation was ca. 2×10^3 CFU/ml for all individual experiments.

Culture conditions

The culture medium used was BHI broth (Difco). This was modified by the addition of NaCl or $NaNO_2$ (filter sterilized) or by adjustment of pH (with HCl). The basal medium contains 0.5 NaCl and is pH 7.3. Incubation was aerobic (shaking at 150 rpm) in triplicate flasks (50 ml per 250-ml flask) of the specific variable combination at different temperatures.

Variables and experimental design

The following variables were studied in conjunction with a modified central composite design: temperature (42,37,28,19,12, and 5°C); pH (7.3 to 5.3, in 0.5 pH unit increments); NaCl (0.5% to 4.5%, in 1% increments); and $NaNO_2$ (0 to 200 μg/ml in 50 μg increments).

Bacteriology

At appropriate intervals during incubation, aliquots of culture media were removed, diluted as needed in 0.1% peptone water, and surface plated with a Spiral Plater (Model D) onto tryptic soy agar (Difco). Plates were counted after 24 to 36 h incubation at 28°C incubation with a Laser Counting System (Spiral Systems).

Data processing

Viable cell counts were converted to $\log_{10}$ and the growth curve data were analyzed by the Gompertz equation (*4*) on a computer utilizing the ABACUS, an iterative nonlinear regression program (W. Damert, Eastern Regional Research Center, USDA. Personal communication). The Gompertz equation along with derived growth kinetics equations are shown in Table 1.

Equation development

Quadratic and cubic polynomial models in terms of temperature, pH, sodium chloride level, and sodium nitrite concentration were calculated for Gompertz B and M values and their transformations using the SAS General Linear Model procedure.

TABLE 1. *Equations for Gompertz parameters and derived growth kinetics values.*

The Gompertz equation is:

$L(t) = A + C \exp\{-\exp(-B(t-M))\}$, where:

L(t) = Log_{10} count of bacteria at time (in h) t, [$\log_{10}$ (CFU/ml)].
A = asymptotic log count of bacteria as time decreases indefinitely (initial level of bacteria, $\log_{10}$ CFU/ml).
C = asymptotic amount of growth that occurs as t increases indefinitely (number of log cycles of growth, $\log_{10}$ [CFU/ml].
B = relative growth rate at M, ($\log_{10}$ [CFU/ml]/h) where
M = the time at which the absolute growth rate is maximal (h).

Derived growth kinetics equations:

exponential growth rate (EGR) = B*C/e [$\log_{10}$(CFU/ml)/h]
generation time (GT) = $\log_{10}$2*e/B*C h
lag phase duration (lag) = M-1/B h
Maximum population density (MPD) = A+C $\log_{10}$ (CFU/ml)

RESULTS AND DISCUSSION

Initial analysis and observations suggested that the growth kinetics of *A. hydrophila* K144 were not influenced by the inoculum size (starting count). Experimental verification supported this conclusion (Table 2). These data (starting count, the Gompertz A value, ranged over 1000-fold) indicated that different starting counts (A values) did not affect lag and generation times, the two parameters of greatest meaning and value to food microbiologists. Based on the data in Table 2 and considerations discussed by Buchanan et al. (*1,2*) and Gibson et al. (*4*), model development concentrated on the Gompertz B and M values.

In the subsequent development of the response surface equations, no-growth data were omitted. Again, as proposed by Buchanan et al. (*1,2*) and Gibson et al. (*4*), the natural log transformation was most effective in describing that data. The actual data used in developing the polynomial response models consisted of 54 growth experiments (usually of triplicate flasks of each variable combination); the data analyzed represented 131 separate growth curves. These data and culture condition combinations supporting growth along with the corresponding Gompertz parameters and observed generation and lag times are given in Table 3. Though both quadratic and cubic polynomial response surface models were generated, it was concluded that the quadratic model best described the data under various experimental conditions for reasons to be discussed below.

To extend the value of the data in Table 3 and as an aid to defining culture conditions of temperature, pH, salt, and nitrite under which the organism can and cannot grow, culture conditions which did not support growth are presented (Table 4). In most instances, the culture conditions listed in Table 4 not only did not support growth, the organism generally died off, i.e., the viable count decreased from a starting count of $\log_{10}$ 3 CFU/ml to < 20 CFU/ml (the lower limit of detection) and remained there for at least two sampling periods. The intervals between sampling periods were a function of culture conditions, especially temperature, with shorter intervals at the higher temperatures. In no instance did any of the culture conditions (experiments) in which the organism had "died off" show growth (visible turbidity) upon continued, extended incubations. The significance of this "die off" is unknown in that these cells may be viable but nonculturable (*11*); the role of viable but nonculturable organisms in food microbiology cannot at present be ascertained. However, combining the observations of growth and no growth culture conditions (Tables 3 and 4), investigators can more accurately predict how *A. hydrophila* will respond to different growth conditions.

TABLE 2. *Effect of inoculum size (A, $\log_{10}$ initial count) on calculated Gompertz parameters (triplicate aerobic flasks of BHI, 0.5% NaCl, pH 7.3, 0 mg/L, $NaNO_2$, 19°C).*

Obsv #	A*	C	B	M	lag, h	GT, h	EGR	MPD
1	1.79±0.14	7.72±0.20	0.123±0.006	14.89±0.52	6.7±1.0	0.45±0.04	0.673±0.006	9.51±0.16
2	2.85±0.03	7.42±0.13	0.127±0.006	14.98±0.38	7.2±0.4	0.43±0.02	0.703±0.003	10.27±0.16
3	3.03±0.13	7.42±0.15	0.123±0.006	14.43±0.45	6.4±0.7	0.45±0.03	0.660±0.040	10.45±0.03
4	3.94±0.0	6.55±0.12	0.143±0.006	13.20±0.98	6.2±1.3	0.44±0.05	0.700±0.090	10.49±0.12

*See Table 1 for abbreviations and calculations.

TABLE 3. *Effect of culture conditions on the calculated values of the Gompertz parameters B and M and on the generation time (GT) and lag time (lag) for the aerobic growth of* A. hydrophila.

Variable set	Culture variables temp.°C	pH	NaCl	NaN_{O2}	Number of replicates	Observed B	Observed M	GT, h	lag, h	MPD
1	5	5.3	0.5	0	1	0.0110	270.3	11.31	179.39	10.50
2	5	5.8	0.5	0	3	0.0323	155.1	3.85	124.14	9.87
3	5	6.3	0.5	50	3	0.0342	143.3	3.64	114.06	10.89
4	5	6.3	0.5	100	1	0.0475	184.5	2.62	163.45	10.18
5	5	6.3	1.5	0	1	0.0701	162.0	1.77	147.73	11.60
6	5	6.3	1.5	50	1	0.0464	162.8	2.68	141.25	10.39
7	5	6.3	1.5	100	1	0.0139	159.1	8.95	87.16	9.27
8	5	6.3	2.5	100	3	0.0030	566.9	41.59	232.45	8.20
9	5	6.8	0.5	0	3	0.0373	144.4	3.33	117.59	11.12
10	5	7.3	0.5	0	3	0.0173	103.5	7.19	45.70	10.51
11	8	7.3	0.5	0	3	0.0363	75.3	3.43	47.75	10.60
12	12	5.3	0.5	0	2	0.0330	97.6	3.77	67.30	10.47
13	12	5.3	1.5	0	1	0.0660	33.5	1.88	18.35	10.40
14	12	5.8	1.5	50	3	0.0888	132.2	1.40	120.94	11.09
15	12	5.8	1.5	150	3	0.0237	629.6	5.25	587.35	11.19
16	12	5.8	3.5	50	2	0.0085	508.2	14.63	390.55	9.40
17	12	6.8	1.5	50	6	0.0740	44.1	1.68	30.58	10.59
18	12	6.8	1.5	150	3	0.0478	62.2	2.60	41.29	10.01
19	12	6.8	3.5	50	3	0.0372	107.0	3.34	80.14	9.53
20	12	6.8	3.5	150	3	0.0214	113.2	5.80	66.54	9.01
21	12	7.3	0.5	0	3	0.1050	30.6	1.18	21.08	10.29
22	12	7.3	2.5	0	1	0.0990	69.5	1.26	59.40	11.27
23	12	7.3	2.5	50	1	0.0550	58.9	2.26	40.72	11.29
24	12	7.3	2.5	100	1	0.0915	68.6	1.36	57.67	11.46
25	12	7.3	2.5	200	1	0.1140	68.6	1.09	59.83	11.37
26	12	7.3	3.5	0	1	0.0267	119.6	4.66	82.15	11.56
27	12	7.3	3.5	50	1	0.0279	118.0	4.46	82.16	11.32
28	12	7.3	3.5	100	1	0.0330	106.5	3.77	76.20	11.31
29	12	7.3	3.5	200	1	0.0268	113.0	4.64	75.69	11.19
30	19	5.3	0.5	0	1	0.0790	21.7	1.57	9.04	10.35
31	19	5.8	2.5	100	3	0.0467	123.6	2.66	102.17	9.76
32	19	6.3	0.5	100	3	0.0758	30.2	1.64	17.01	9.18
33	19	6.3	2.5	0	3	0.0476	43.1	2.61	22.08	9.68
34	19	6.3	2.5	100	3	0.0310	65.3	4.01	33.04	10.02
35	19	6.3	2.5	200	3	0.0216	78.1	5.77	31.74	10.47
36	19	7.3	0.5	0	3	0.1860	11.1	0.67	5.72	10.56
37	19	7.3	2.5	100	3	0.0574	40.4	2.17	22.99	9.94
38	24	7.3	0.5	0	3	0.3110	11.0	0.40	7.78	10.61
39	28	5.3	0.5	0	3	0.2113	9.1	0.59	4.37	10.23
40	28	5.3	1.5	0	1	0.1360	12.0	0.91	4.65	10.36
41	28	5.8	1.5	50	3	0.1607	11.7	0.77	5.48	8.98
42	28	5.8	1.5	150	3	0.0685	38.1	1.82	23.50	7.96
43	28	5.8	3.5	50	3	0.0288	74.0	4.32	39.28	8.46
44	28	5.8	3.5	150	3	0.1015	46.8	1.23	36.94	5.41
45	28	6.8	1.5	50	6	0.2172	9.6	0.57	5.00	10.45
46	28	6.8	1.5	150	3	0.2073	9.7	0.60	4.88	9.95
47	28	6.8	3.5	50	3	0.3657	23.2	0.34	20.47	9.20
48	28	6.8	3.5	150	6	0.1346	33.6	0.92	26.17	9.63
49	28	7.3	0.5	0	3	0.4133	6.4	0.30	3.98	10.17
50	37	5.8	0.5	0	3	0.2667	6.7	0.47	2.95	8.90
51	37	7.3	0.5	0	3	0.2733	4.8	0.45	1.14	10.07
52	37	7.3	0.5	100	1	0.4010	6.0	0.31	3.51	9.32
53	37	7.3	0.5	200	1	0.3640	6.3	0.34	3.55	9.87
54	42	7.3	0.5	0	3	0.0987	51.8	1.26	41.67	8.47

TABLE 4. *Conditions of temperature, NaCl, pH, and $NaNO_2$ which did not support the aerobic growth* of* A. hydrophila.

Temp, °C	%NaCl	$NaNO_2$ mg/L	pH
5	1.5	0	5.3
12	0.5	150	5.3
12	3.5	150	5.8
19	2.5	100	5.3
19	4.5	100	6.3
37	0.5	0	5.3
37	1.5	0	5.3
37	0.5	50	5.8
37	0.5	100	5.8
37	0.5	150	5.8
37	0.5	200	5.8
37	4.5	0	5.8
37	4.5	200	5.8
42	0.5	0	5.3
42	1.5	0	5.3
42	2.5	100	6.3

*See text for definition of no growth.

Before determining which model better fits and describes the data in Table 3, an overview of the results can be made. This is one of the first studies on the effect of nitrite on the growth of *A. hydrophila*. As with other bacteria, nitrite is most effective at inhibiting the organism in media at pH values below 6.0. Nitrite when combined with 2.5 to 3.5% NaCl, low temperatures (5 or 12°C), and low pH values (6.3 or lower) is particularly effective in controlling the growth of *A. hydrophila*. Individually, the culture variables do not greatly influence growth kinetics. Based on generation time (GT), the optimum temperature for *A. hydrophila* K144 is 28°; however, lag time is shortest at 37°. Popoff *(10)* stated that the optimum temperature for the genus *Aeromonas* is 22-28°C, with members of the mesophilic group (*A. hydrophila, A. sobria,* and *A. caviae*) capable of growth at 37°C. Previously, Palumbo et al. *(8)* reported better performance of starch ampicillin agar for isolating mesophilic aeromonads from foods of animal origin when incubated at 28 vs. 37°C.

To develop the predictive models (equations) for the Gompertz B and M values, growth data (Table 3) were subjected to response surface analysis using SAS's general linear model. The resulting second and third order polynomial equations for B and M are given in Table 5.

In ascertaining which model (quadratic vs. cubic) better describes the data, several different approaches and analyses can be employed. R^2 values can be used to compare the actual values of B and M with those predicted by the two different models. These data are given in Table 6. It can be seen that as the level of the model increases from second to third order, so do the R^2 values indicating a better fit with the cubic model.

TABLE 6. *Comparison of fit (R^2 values) for the quadratic and cubic predictive models generated for B and M.*

Gompertz parameter	Model quad	cubic
B	0.747[a]	0.900[b]
M	0.908[c]	0.973[d]

[a]Max R^2 = 0.958.
[b]Max R^2 = 0.957.
[c]Max R^2 = 0.998.
[d]Max R^2 = 0.997.

The second approach to evaluating and comparing the quadratic and cubic models was to use the corresponding equations to generate predicted values for B and M for each culture variation listed in Table 3. These data are presented in Table 7. From the predicted B and M values and using a value of 6.58 for C, predicted values for lag time and GT were also generated for each culture variable combination for each model. In general, the cubic equations and their predicted B and M values yielded GT and lag statistically closer to the observed values. This was to be expected since the R^2 values (Table 6) were observed to increase as the model level increased from quadratic to cubic. R^2 represents the difference between observed values and predicted ones by each of the models, with a higher R^2 value indicating a better agreement of predicted to observed.

With the last method of comparing and evaluating the two models, various culture condition combinations not employed in developing the equations were incorporated into the individual equations and corresponding values for GT and lag times generated. These data are presented in Table 8. It can be seen that for certain combinations of culture conditions with the cubic model, GTs of 0.0 (data lines 2 and 9) and negative lag times (data lines 14, 18, and 23) are obtained; this is not seen in the case of the quadratic model.

In conclusion, this study represents the first systematic investigation of the influence of temperature, pH, sodium chloride, and sodium nitrite on the kinetics of aerobic growth of *A. hydrophila*. The results of this study should be applicable to virtually any food for which pH, NaCl and nitrite level, and storage temperature are known. Besides conditions which supported growth, we also obtained values for the lag and generation times as influenced by culture conditions (Table 3). The kinetic parameters were then analyzed by response surface techniques and second and third order polynomial equations in the four variables were generated for the Gompertz B and M values (Table 5). The adequacy of these predictive models was tested against both the culture conditions used to generate them and a theoretical set of culture variables within the data ranges used. When tested against the culture conditions used to generate them, it was observed that the cubic model (equation) yielded the better fit statistically (Tables 6 and 7). When these predictive equations were tested with an addi

TABLE 5. *Response surface models in temperature (°C), pH, NaCl (%), and $NaNO_2$ (mg/L) for the Gompertz parameters B and M for aerobic growth of* A. hydrophila.

Second Order equations:

$$\begin{aligned}\text{Ln (B)} = &- 14.126 + 0.214*\text{temp} + 3.065*\text{pH} - 1.952*\text{NaCl} - 0.0234*\text{NaNO}_2 \\ &- 0.0035*\text{temp}*\text{pH} + 0.0127*\text{temp}*\text{NaCl} + 0.000085*\text{temp}*\text{NaNO}_2 \\ &+ 0.2097*\text{pH}*\text{NaCL} + 0.00299*\text{pH}*\text{NaNO}_2 - 0.000156*\text{NaCl}*\text{NaNO}_2 \\ &- 0.0032*\text{temp}*\text{temp} - 0.2315*\text{pH}*\text{pH} - 0.0016*\text{NaCl}*\text{NaCl} \\ &- 0.00000329*\text{NaNO}_2*\text{NaNO}_2\end{aligned}$$

$$\begin{aligned}\text{Ln(M)} = &\ 29.04 - 0.4397*\text{temp} - 6.651*\text{pH} + 0.4744*\text{NaCl} + 0.0597*\text{NaNO}_2 \\ &+ 0.0254*\text{temp}*\text{pH} + 0.00546*\text{temp}*\text{NaCl} - 0.000249*\text{temp}*\text{NaNO}_2 \\ &- 0.0317*\text{pH}*\text{NaCl} - 0.00752*\text{pH}*\text{NaNO}_2 - 0.00185*\text{NaCl}*\text{NaNO}_2 \\ &+ 0.0044*\text{temp}*\text{temp} + 0.472*\text{pH}*\text{pH} + 0.0564*\text{NaCl}*\text{NaCl} \\ &+ 0.00001*\text{NaNO}_2*\text{NaNO}_2\end{aligned}$$

Third Order equations:

$$\begin{aligned}\text{Ln(B)} = &29.9712 + 1.263*\text{temp} - 23.5454*\text{pH} + 11.668*\text{NaCl} + 0.1387*\text{NaNO}_2 \\ &- 0.4675*\text{temp}*\text{pH} - 0.1658*\text{temp}*\text{NaCl} + 0.004193*\text{temp}*\text{NaNO}_2 \\ &- 1.6288*\text{pH}*\text{NaCl} - 0.06466*\text{pH}*\text{NaNO}_2 + 0.4115*\text{NaCl}*\text{NaNO}_2 \\ &+ 0.0203*\text{temp}*\text{temp} + 4.7927*\text{pH}*\text{pH} - 3.8508*\text{NaCl}*\text{NaCl} \\ &- 0.0002353*\text{NaNO}_2*\text{NaNO}_2 + 0.02935*\text{temp}*\text{pH}*\text{NaCl} \\ &- 0.0006*\text{temp}*\text{pH}*\text{NaNO}_2 + 0.00187*\text{temp}*\text{NaCl}*\text{NaNO}_2 \\ &- 0.00788*\text{pH}*\text{NaCl}*\text{NaNO}_2 - 0.00266*\text{temp}*\text{temp}*\text{pH} \\ &- 0.002217*\text{temp}*\text{temp}*\text{NaCl} + 0.00000577*\text{temp}*\text{temp}*\text{NaNO}_2 \\ &+ 0.04712*\text{pH}*\text{pH}*\text{NaCl} + 0.00651*\text{pH}*\text{pH}*\text{NaNO}_2 + 0.0436*\text{pH}*\text{pH}*\text{temp} \\ &+ 0.000302*\text{NaCl}*\text{NaCl}*\text{NaNO}_2 + 0.2806*\text{NaCl}*\text{NaCl}*\text{pH} \\ &+ 0.01575*\text{NaCl}*\ \text{NaCl}*\ \text{temp} - 0.00000552*\text{NaNO}_2*\text{NaNO}_2*\text{temp} \\ &+ 0.00004636*\text{NaNO}_2*\text{NaNO}_2*\text{pH} - 0.00003665*\text{NaNO}_2*\text{NaNO}_2*\text{NaCl} \\ &- 0.00007265*\text{temp}*\text{temp}*\text{temp} - 0.30606*\text{pH}*\text{pH}*\text{pH} \\ &+ 0.26559*\text{NaCl}*\text{NaCl}*\text{NaCl} + 0.00000043*\text{NaNO}_2*\text{NaNO}_2*\text{NaNO}_2\end{aligned}$$

$$\begin{aligned}\text{Ln(M)} = &\ 38.195 + 0.7536*\text{temp} - 15.952*\text{pH} - 8.0078*\text{NaCl} + 0.5475*\text{NaNO}_2 \\ &- 0.2163*\text{temp}*\text{pH} + 0.02779*\text{temp}*\text{NaCl} - 0.003509*\text{temp}*\text{NaNO}_2 \\ &+ 0.9828*\text{pH}*\text{NaCl} - 0.1389*\text{pH}*\text{NaNO}_2 - 0.02967*\text{NaCl}*\text{NaNO}_2 \\ &- 0.0174*\text{temp}*\text{temp} + 2.711*\text{pH}*\text{pH} + 2.9066*\text{NaCl}*\text{NaCl} \\ &- 0.00006681*\text{NaNO}_2*\text{NaNO}_2 - 0.00945*\text{temp}*\text{pH}*\text{NaCl} \\ &+ 0.000678*\text{temp}*\text{pH}*\text{NaNO}_2 - 0.00000377*\text{temp}*\text{NaCl}*\text{NaNO}_2 \\ &+ 0.0054*\text{pH}*\text{NaCl}*\text{NaNO}_2 + 0.00058*\text{temp}*\text{temp}*\text{pH} \\ &+ 0.002451*\text{temp}*\text{temp}*\text{NaCl} - 0.00002461*\text{temp}*\text{temp}*\text{NaNO}_2 \\ &- 0.009194*\text{pH}*\text{pH}*\text{NaCl} + 0.00807*\text{pH}*\text{pH}*\text{NaNO}_2 + 0.0164*\text{pH}*\text{pH}*\text{temp} \\ &- 0.0015*\text{NaCl}*\text{NaCl}*\text{NaNO}_2 - 0.2304*\text{NaCl}*\text{NaCl}*\text{pH} \\ &- 0.01232*\text{NaCl}*\text{NaCl}*\text{temp} + 0.0000003*\text{NaNO}_2*\text{NaNO}_2*\text{temp} \\ &+ 0.00002264*\text{NaNO}_2*\text{NaNO}_2*\text{pH} - 0.00000221*\text{NaNO}_2*\text{NaNO}_2*\text{NaCl} \\ &+ 0.00025543*\text{temp}*\text{temp}*\text{temp} - 0.15726*\text{pH}*\text{pH}*\text{pH} \\ &- 0.17907*\text{NaCl}*\text{NaCl}*\text{NaCl} - 0.00000028*\text{NaNO}_2*\text{NaNO}_2*\text{NaNO}_2\end{aligned}$$

TABLE 7. *Effect of culture conditions for the aerobic growth of* A. hydrophila *on values of the Gompertz parameters B and M and generation (GT) and lag times predicted by the quadratic and cubic models. (Used C = 6.58, average from all aerobic experiments in which growth occured).*

Variable	Predicted B		Predicted M		Predicted GT		Predicted lag	
set	quad	cubic	quad	cubic	quad	cubic	quad	cubic
1	0.0208	0.0188	333.3	189.7	5.99	6.61	285.15	136.51
2	0.0278	0.0303	173.7	172.2	4.47	4.10	137.72	139.24
3	0.0267	0.0262	196.0	164.5	4.66	4.75	158.49	126.29
4	0.0211	0.0236	352.2	200.1	5.89	5.28	304.83	157.64
5	0.0187	0.0503	173.6	149.1	6.64	2.47	120.24	129.21
6	0.0150	0.0263	270.5	162.4	8.31	4.73	203.66	124.36
7	0.0118	0.0167	443.0	207.5	10.58	7.46	375.89	147.48
8	0.0065	0.0043	623.8	516.5	19.07	29.06	470.43	282.86
9	0.0352	0.0406	95.6	136.4	3.53	3.06	67.17	111.78
10	0.0333	0.0213	101.3	94.1	3.74	5.85	71.24	47.09
11	0.0527	0.0375	56.6	70.5	2.36	3.32	37.64	43.84
12	0.0583	0.0325	67.9	87.8	2.13	3.82	50.76	57.06
13	0.0292	0.0501	110.3	54.4	4.25	2.48	76.10	34.42
14	0.0328	0.0467	106.6	116.7	3.79	2.66	76.08	95.28
15	0.0182	0.0203	367.8	670.0	6.84	6.14	312.76	620.64
16	0.0099	0.0088	317.2	460.8	12.55	14.20	216.29	346.64
17	0.0580	0.0817	46.7	44.5	2.14	1.52	29.46	32.26
18	0.0434	0.0756	76.0	49.0	2.87	1.65	52.95	35.77
19	0.0267	0.0294	103.4	135.8	4.66	4.23	65.90	101.82
20	0.0193	0.0168	146.5	123.7	6.43	7.40	94.79	64.21
21	0.0890	0.0754	29.4	41.1	1.40	1.65	18.17	27.84
22	0.0516	0.0476	76.6	87.4	2.41	2.61	57.23	66.39
23	0.0489	0.0350	68.4	71.3	2.54	3.55	47.96	42.76
24	0.0456	0.0271	64.3	76.4	2.73	4.60	42.38	39.43
25	0.0378	0.0673	66.0	85.7	3.29	1.85	39.52	70.84
26	0.0391	0.0531	146.2	98.2	3.18	2.34	120.64	79.38
27	0.0368	0.0436	119.1	82.5	3.38	2.85	91.92	59.55
28	0.0340	0.0312	102.0	90.0	3.65	3.98	72.62	57.96
29	0.0277	0.0386	87.0	101.3	4.48	3.22	50.95	75.37
30	0.1195	0.0715	21.3	27.7	1.04	1.74	12.93	13.71
31	0.0345	0.0340	89.4	127.2	3.61	3.66	60.40	97.75
32	0.1302	0.0872	22.7	30.7	0.96	1.43	15.02	19.24
33	0.0827	0.0559	31.3	39.3	1.50	2.23	19.21	21.40
34	0.0575	0.0387	46.9	59.4	2.16	3.22	29.50	33.54
35	0.0374	0.0214	85.7	76.2	3.32	5.81	58.97	29.47
36	0.1737	0.2015	13.2	13.7	0.72	0.62	7.44	8.74
37	0.1129	0.0987	26.1	40.3	1.10	1.26	17.24	30.17
38	0.2307	0.3149	9.7	7.1	0.54	0.39	5.37	3.92
39	0.1887	0.2199	9.1	8.3	0.66	0.57	3.80	3.75
40	0.1159	0.1151	16.1	11.7	1.07	1.08	7.47	3.01
41	0.1352	0.1584	15.6	13.2	0.92	0.79	8.21	6.89
42	0.0858	0.0831	36.2	33.7	1.45	1.50	24.55	21.66
43	0.0614	0.0337	55.8	69.9	2.02	3.70	38.92	40.18
44	0.0378	0.0958	88.7	49.6	3.29	1.30	62.24	39.16
45	0.2265	0.2337	10.3	8.6	0.55	0.53	5.88	4.32
46	0.1939	0.1000	11.2	12.5	0.64	1.24	6.04	2.50
47	0.1565	0.2439	34.2	26.1	0.79	0.51	27.81	22.00
48	0.1299	0.1172	25.9	30.9	0.96	1.06	18.20	22.36
49	0.2578	0.3702	8.9	5.2	0.48	0.34	5.02	2.50
50	0.2238	0.2533	6.2	7.0	0.56	0.49	1.73	3.05
51	0.2269	0.2425	12.2	9.3	0.55	0.51	7.79	5.18
52	0.2556	0.3173	8.0	5.8	0.49	0.39	4.09	2.65
53	0.2696	0.4978	6.4	5.9	0.46	0.25	2.69	3.89
54	0.1686	0.1092	20.0	37.4	0.74	1.14	14.07	28.24

*See Table 3 for culture variables corresponding to the variable sets.

TABLE 8. *Comparison of GT and lag predicted from quadratic and cubic models for various variable combinations.*

Culture conditions				Quad		Cubic	
temp, °C	NaCl	pH	$NaNO_2$	GT, h	lag, h	GT, h	lag, h
5	0.5	7.0	0	4.0	66.3	4.2	89.1
5	0.5	7.0	200	7.0	334.5	0.0	8.7
5	2.5	7.0	0	9.4	166.1	15.0	287.0
5	0.5	6.0	0	4.7	109.7	4.0	137.4
5	0.5	6.0	200	14.8	2495.2	2.2	1308.7
5	3.5	6.0	200	109.2	3515.9	53.9	1028.7
10	0.5	5.5	0	2.8	58.9	4.4	74.2
10	0.5	6.5	0	1.9	23.3	2.0	68.2
10	0.5	7.0	200	3.1	92.3	0.0	12.2
10	3.5	7.0	200	10.0	114.8	8.6	53.4
19	1.5	6.0	100	2.1	29.2	2.2	38.1
19	1.5	5.5	100	3.3	71.4	2.0	168.1
19	0.5	7.0	0	0.8	5.7	0.7	10.3
28	3.5	6.0	0	1.5	32.1	13.5	-28.8
28	3.5	6.0	200	3.6	56.5	2.1	6.6
37	0.5	5.5	0	0.7	1.9	0.4	5.3
37	0.5	5.5	100	1.1	9.9	0.2	12.5
37	0.5	6.5	100	0.6	3.4	0.7	-1.6
37	3.5	6.5	100	0.9	29.6	3.3	133.2
37	3.5	7.0	100	0.6	28.3	1.4	213.3
37	0.5	7.0	100	0.6	3.4	0.6	0.0
37	0.5	7.0	0	0.6	4.9	0.6	3.4
37	0.5	7.0	200	0.6	3.3	0.6	-1.1
42	0.5	7.0	0	0.8	8.7	1.3	19.3

tional set of conditions (the so-called "use test"), the cubic model gave several examples of nonsense, e.g., negative lag times or generation times of zero (Table 8). The "nonsense" regions in Table 8 might be eliminated by further experimentation, i.e., variable combinations in these regions. However, the "nonsense" regions in many instances are close to nongrowth conditions and thus growth would be extremely slow and the growth kinetics less dependable. If data were obtained for these regions, the more statistically reliable cubic model could undoubtedly be used. Thus, despite strong statistical validation for the cubic models [predicted values for B and M (Table 7) and R^2 values (Table 6)], the quadratic equations performed better in the "use test" (Table 8) and represent the model level which should be used to analyze the influence of temperature, pH, sodium chloride, and sodium nitrite on the aerobic growth of *A. hydrophila*. These results also indicate that the aerobic growth of *A. hydrophila* at low incubation temperatures can only be controlled by a combination of low pH and high salt and nitrite levels.

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